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Peripheral association of a polyprotein precursor form of the RNA-dependent RNA polymerase of *Tomato ringspot virus* with the membrane-bound viral replication complex

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Abstract

Replication of *Tomato ringspot virus* (ToRSV) occurs in association with endoplasmic reticulum (ER)-derived membranes. We have previously shown that the putative nucleotide triphosphate-binding protein (NTB) of ToRSV is an ER-targeted protein and that an intermediate polyprotein containing the domains for NTB and for the genome-linked viral protein (VPg) is associated with the replication complex. We now report the detection of a 95-kDa polyprotein that contains the domains for the RNA-dependent RNA polymerase (Pol), the proteinase (Pro) and the VPg. This polyprotein appears to be a truncated version of the full-length 111-kDa VPg-Pro-Pol polyprotein and was termed VPg-Pro-Pol′. A subpopulation of VPg-Pro-Pol′ was peripherally associated with ER-derived membranes active in viral replication. However, the VPg, Pro and Pol domains did not target to membranes in the absence of viral infection. We propose a model in which VPg-Pro-Pol′ is brought to the site of replication through interaction with a viral membrane protein.

Keywords: Positive-strand RNA virus; RNA replication; RNA-dependent RNA polymerase; Picorna-like virus; Nepovirus; Comoviridae; Membrane association; Replication complex

Introduction

Replication of positive-strand RNA viruses occurs in association with intracellular membranes (Salonen et al., 2005). Proliferation of membranous vesicles or spherules is a common cytopathological effect that results from infection by positive-strand RNA viruses. These membranous structures constitute subcellular compartments in which active viral replication takes place. These structures allow a local concentration of replication proteins and protection of the viral RNA from degradation (Schwartz et al., 2002). The membrane-bound replication complex includes viral proteins, host proteins and the viral RNA (reviewed in Boguszewska-Chachulska and Haenni, 2005; Noueiry and Ahlquist, 2003; Sanfacon, 2005; White and Nagy, 2004). The mechanisms by which the replication complex is assembled are still relatively poorly understood. For some viruses, specific viral replication proteins have been proposed to act as membrane anchors for the replication complex and probably play a key role in organizing the assembly of the complex. These proteins interact directly with the membranes involved in replication. They also interact with other viral replication proteins that do not possess membrane-targeting domains and redirect these proteins to the site of replication. For example, the 1a protein of *Brome mosaic virus* redirects the 2a RNA-dependent RNA polymerase (Pol) and the viral RNA to endoplasmic reticulum (ER) membranes, the site of viral replication (Chen and Ahlquist, 2000; Wang et al., 2005). Similarly, the *Cucumber necrosis virus* p33 protein is an integral membrane protein that orchestrates the formation of

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the replication complex in association with peroxisomal membranes and redirects the Pol and viral RNA to this complex (Panavas et al., 2005). Also, the 140-kDa protein of *Turnip yellow mosaic virus* recruits the viral Pol to chloroplast membranes, where replication complexes are assembled (Jakubiec et al., 2004). For viruses that express their proteins using a polyprotein strategy, viral replication proteins can be brought to the replication complex as intermediate polyproteins that include the viral membrane anchor. This has been shown for the NIa protein of potyviruses, which contains the domains for the genome-linked VPg protein and the proteinase (Pro). While the NIa protein is transported into the nucleus, a larger polyprotein (6K-NIa) that also includes the hydrophobic 6-kDa protein is targeted to ER membranes (Restrepo-Hartwig and Carrington, 1994). In many cases, a combination of strategies is used. Such is the case for the NIb polymerase of potyviruses, which is probably brought to the site of replication through protein–protein interaction with the Pro domain of the 6K-NIa polyprotein (Li et al., 1997). Also, assembly and/or maintenance of the poliovirus (PV) replication complex requires interaction between the 3CD (Pro-Pol) and 3D (Pol) soluble proteins and the 3AB, 2BC, 2B and/or 2C membrane-bound proteins (Agol et al., 1999).

Tomato ringspot virus (ToRSV, genus *Nepovirus*, family Comoviridae) has a bipartite genome. Each RNA molecule is translated into one large polyprotein that is cleaved by the viral-encoded proteinase into smaller intermediate polyproteins and mature proteins (Sanfacon et al., 2006). RNA1 encodes the replication proteins including a putative nucleotide triphosphate-binding protein (NTB), the VPg, a 3C-like Pro and the Pol (Rott et al., 1995). The proteinase cleavage sites that separate these domains have been identified (Wang et al., 1999; Wang and Sanfacon, 2000). The RNA1-encoded polyprotein also contains two additional protein domains, X1 and X2 of unknown function (Wang and Sanfacon, 2000). In ToRSV-infected plant cells, viral replication occurs in association with ER-derived membranes (Han and Sanfacon, 2003). The NTB and X2 proteins of ToRSV are integral membrane proteins that associate with the ER when they are expressed independently of other viral proteins (Zhang and Sanfacon, 2006; Zhang et al., 2005), suggesting that one or both of these proteins function as the membrane anchor for the replication complex. In the case of NTB, this suggestion is supported by the observation that both the mature NTB and the intermediate NTB-VPg polyprotein are found in association with ER-derived membranes active in viral replication (Han and Sanfacon, 2003). While the association of membrane proteins containing the NTB domain with the replication complex has been established, the mechanisms by which other viral replication proteins (e.g., Pro and Pol) are brought into the complex are not known.

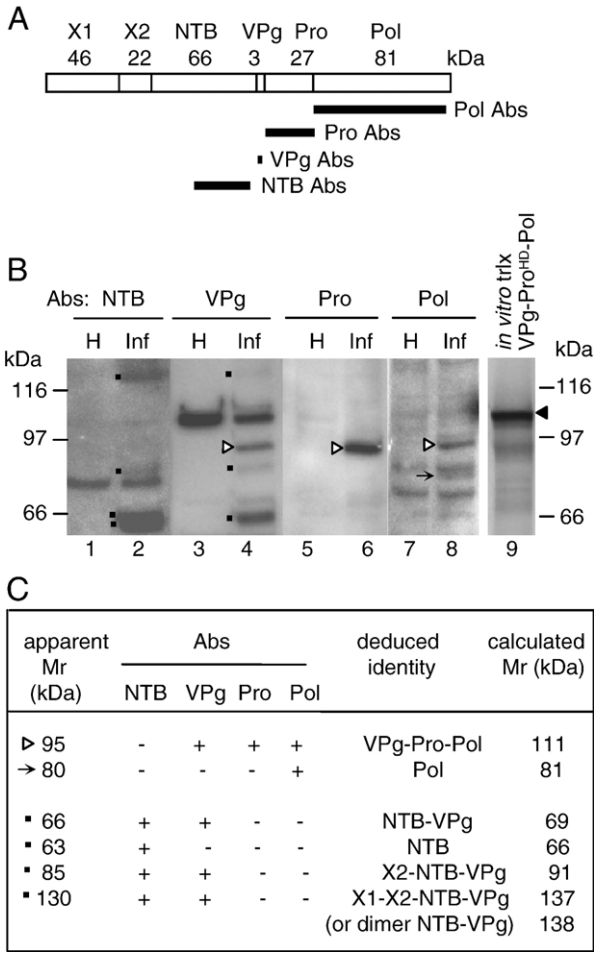
In this study, we report the detection of a 95-kDa intermediate polyprotein containing the VPg, Pro and Pol domains in extracts prepared from ToRSV-infected cucumber. This protein is likely a truncated version of the full-length 111-kDa VPg-Pro-Pol polyprotein and was termed VPg-Pro-Pol′. A subpopulation of the VPg-Pro-Pol′ polyprotein is membrane-associated and co-fractionates with ER-derived membranes active in viral replication. Further experiments demonstrate that the association of VPg-Pro-Pol′ with membranes is peripheral. Because the VPg, Pro and Pol domains do not associate with ER-membranes in the absence of viral infection, we propose a model in which VPg-Pro-Pol′ is brought into the replication complex through interaction with one or several viral membrane proteins.

Results

Immunodetection of a 95-kDa VPg-Pro-Pol polyprotein in ToRSV-infected cucumber extracts

To detect mature proteins or intermediate polyproteins containing the Pro and Pol domains, we prepared rabbit polyclonal antibodies using purified recombinant proteinase (Chisholm et al., 2001) or recombinant polymerase as antigen (see Materials and methods). In addition, we also used two previously described antibodies specific for the NTB and VPg domains (Han and Sanfacon, 2003) (see Fig. 1A for a graphical representation of the protein domains against which each antibody was raised). Immunoblotting experiments were conducted using post-nuclear extracts (S3 fractions) prepared from ToRSV-infected or healthy cucumber cotyledons. To prevent degradation of the proteins during the extraction process, protease inhibitors were added to the extraction buffer (see Materials and methods). Several proteins were detected in extracts prepared from ToRSV-infected plants that were not present in the control extracts derived from healthy plants (Fig. 1B). The NTB antibodies recognized a predominant doublet of proteins having apparent molecular masses of 66 and 63 kDa that correspond to the NTB-VPg polyprotein and the mature NTB, respectively (see Fig. 1C for a summary of the properties of each protein and their deduced identity). An 85-kDa protein was recognized by the NTB and VPg antibodies but not by the Pro antibodies, suggesting that it is an intermediate polyprotein containing the X2, NTB and VPg domains. A 130-kDa protein was detected by the NTB and VPg antibodies but not by the Pro or Pol antibodies and could be either a large polyprotein containing the X1, X2, NTB and VPg domains or a dimeric form of NTB-VPg and/or NTB. These results were similar to our previous observations (Han and Sanfacon, 2003).

Using the Pro antibodies, a predominant protein of approximately 95 kDa was detected. This protein was also detected by the VPg and Pol antibodies but not by the NTB antibodies, suggesting that it is an intermediate polyprotein containing the VPg, Pro and Pol domains. A protein of approximately 80 kDa was occasionally detected by the Pol antibodies but not by any of the other antibodies tested. This protein likely corresponded to the mature polymerase. Finally, smaller proteins were also occasionally detected with the Pol antibodies with apparent molecular masses of 53 kDa and 28 kDa (data not shown) and may represent cleavage products derived from the Pol. The reproducible detection of the 95-kDa VPg-Pro-Pol polyprotein in infected cucumber extracts suggested that it is a stable intermediate polyprotein. Interestingly, the estimated molecular mass of this protein based on its



C

apparent Mr (kDa)	Abs NTB	VPg	Pro	Pol	deduced identity	calculated Mr (kDa)
▷ 95	-	+	+	+	VPg-Pro-Pol	111
→ 80	-	-	-	+	Pol	81
▪ 66	+	+	-	-	NTB-VPg	69
▪ 63	+	-	-	-	NTB	66
▪ 85	+	+	-	-	X2-NTB-VPg	91
▪ 130	+	+	-	-	X1-X2-NTB-VPg (or dimer NTB-VPg)	137 138

Fig. 1. Immunodetection of viral replication proteins in cucumber extracts. (A) Schematic representation of the genomic organization of the ToRSV RNA1-encoded polyprotein. The open boxes represent the individual protein domains. The predicted molecular mass of each protein domain is indicated above the boxes. The vertical lines represent the proteinase cleavage sites. The regions of the RNA1-encoded polyprotein against which each antibody was raised are indicated by the thick black lines below the boxes. (B) Immunoblot detection of viral replication proteins in healthy (H) and ToRSV-infected (Inf) S3 extracts. Lanes 1–8: Cucumber tissue was extracted and the proteins were separated by SDS–PAGE (8% polyacrylamide), transferred to PVDF membranes and probed with antibodies raised against the NTB, VPg, Pro and Pol domains as indicated above each lane. Migration of molecular mass standards is indicated on the left. Small black boxes show proteins recognized by the NTB antibodies. The open arrow-head points to the putative truncated VPg-Pro-Pol precursor and the black arrow indicates the mature polymerase which is recognized only by the Pol antibodies. Lane 9: The full-length VPg-Pro^{HD}-Pol polyprotein was synthesized using a rabbit reticulocyte translation system (*in vitro* trlx) and separated by SDS–PAGE (8% polyacrylamide). Migration of molecular mass standards is shown on the right. The black arrowhead indicates the full-length VPg-Pro-Pol polyprotein. (C) Deduced identity of viral replication proteins detected by immunoblotting of S3 extracts derived from ToRSV-infected plants.

migration in SDS–PAGE was smaller than the molecular mass predicted for the full-length VPg-Pro-Pol intermediate polyprotein (111 kDa). This prediction is based on the previously identified NTB-VPg cleavage site (Wang et al., 1999) and on the position of the stop codon at the 3′end of the Pol coding region. The estimated molecular mass of the detected VPg-Pro-Pol polyprotein suggested that it migrated aberrantly in SDS–polyacrylamide gels or that it was truncated. To test this, we synthesized the full-length VPg-Pro-Pol polyprotein *in vitro* in a coupled transcription–translation reaction. To prevent proteolytic cleavage of the VPg-Pro-Pol precursor, a previously described mutation was introduced in the putative catalytic triad ($H^{1283}D$) of the proteinase (Hans and Sanfacon, 1995). The VPg-Pro^{HD}-Pol polyprotein synthesized *in vitro* migrated to a position corresponding to that expected for the full-length polyprotein (~110 kDa; Fig. 1B, lane 9), suggesting that the 95-kDa polyprotein detected *in vivo* is a truncated version of this polyprotein. Since the N-terminal VPg domain of the 95-kDa polyprotein is detected with the VPg antibody, the missing fragment is likely from the C-terminus of the Pol domain. Hereafter we refer to the 95-kDa polyprotein as VPg-Pro-Pol′.

In vitro processing of the VPg-Pro-Pol and NTB-Pol polyproteins: evidence that VPg-Pro-Pol is a stable precursor

To determine if VPg-Pro-Pol is a stable intermediate, we examined the processing of the VPg-Pro-Pol polyprotein *in vitro*. The wild-type VPg-Pro-Pol polyprotein (Fig. 2A) was synthesized *in vitro* as above and incubated at 16 °C overnight to allow proteolytic processing to occur. As a control, the VPg-Pro^{H1283D}-Pol mutant derivative was also tested. After 16 h incubation, the *in vitro* synthesized wild-type VPg-Pro-Pol was partially processed resulting in the release of a 32-kDa protein that probably corresponds to an intermediate polyprotein containing the VPg-Pro domains following proteolysis at the Pro-Pol cleavage site (Fig. 2B, lane 1). Surprisingly, the mature Pol was not detected in the processing reaction of the wild-type VPg-Pro-Pol even when the translation products were separated in gels of lower acrylamide concentrations (Fig 2B, lane 3). The reasons for this are not obvious but it suggests that the mature Pol is unstable. The inefficient processing of VPg-Pro-Pol after prolonged incubation suggested that it was a stable intermediate at least *in vitro*.

To further test the stability of the VPg-Pro-Pol intermediate *in vitro*, we next examined the cleavage of a larger polyprotein precursor that included the NTB, VPg, Pro and Pol domains (NTB-Pol polyprotein, Fig. 2C). As above, we compared the cleavage products of the wild-type polyprotein with those of a mutant derivative that contained an inactive proteinase (HD mutant as above). After a 16-h incubation period, most of the NTB-Pol polyprotein was processed to release three major cleavage products: (1) a 110-kDa protein, which is the expected molecular mass for the VPg-Pro-Pol intermediate polyprotein; (2) a 63-kDa protein, which corresponds to the mature NTB protein as previously confirmed by immunoprecipitation using NTB antibodies (Wang and Sanfacon, 2000); and (3) a 32-kDa protein, which was previously identified as the VPg-Pro polyprotein (Wang and Sanfacon, 2000) (Fig. 2D, lane 1). As above, the mature Pol was not observed in the cleavage products. To confirm the nature of the cleavage products, point mutations were introduced in the NTB-VPg and Pro-Pol cleavage sites. The conserved glutamine at the −1 position of the cleavage sites was deleted. We have previously determined that the presence of a glutamine at the −1 position is an absolute

requirement for processing at ToRSV cleavage sites (Carrier et al., 1999). Mutation of the NTB-VPg cleavage site drastically changed the pattern of the cleavage products that were produced [see ΔQ (NV) mutant, Figs. 2C and D, lane 3]. The NTB-Pol-ΔQ (NV) polyprotein was cleaved only inefficiently compared to the wild-type polyprotein as indicated by the presence of a large amount of the polyprotein remaining after the 16-h incubation. As expected, the VPg-Pro-Pol, NTB and VPg-Pro cleavage products were not detected. Instead, small amounts of a 66-kDa protein (likely corresponding to the NTB-VPg cleavage product) and a 28-kDa protein (with an estimated molecular mass consistent with that expected for the mature Pro) were observed. Mutation of the Pro-Pol cleavage site had a much less drastic effect on the cleavage of the NTB-Pol polyprotein [see ΔQ (PP) mutant, Figs. 2C and D, lane 4]. The NTB-Pol-ΔQ (PP) polyprotein was efficiently processed to release the VPg-Pro-Pol polyprotein and the mature NTB protein. As expected, the VPg-Pro cleavage product was not detected. Finally, simultaneous mutation of the NTB-VPg and Pro-Pol cleavage sites completely prevented cleavage of the NTB-Pol polyprotein [see ΔQ (NV+PP) mutant; Fig. 2D, lane 5]. Taken together, these results suggest that cleavage of the wild-type NTB-Pol precursor *in vitro* occurs predominantly at the NTB-VPg cleavage site. Accumulation of the VPg-Pro-Pol intermediate polyprotein in the cleavage products of the wild-type NTB-Pol polyprotein also supports the suggestion that it is a stable intermediate *in vitro*. This result is consistent with the detection of a polyprotein containing the VPg, Pro and Pol domains *in vivo*. However, *in vivo* the VPg-Pro-Pol protein is apparently further cleaved at an additional site in the C-terminal region of Pol.

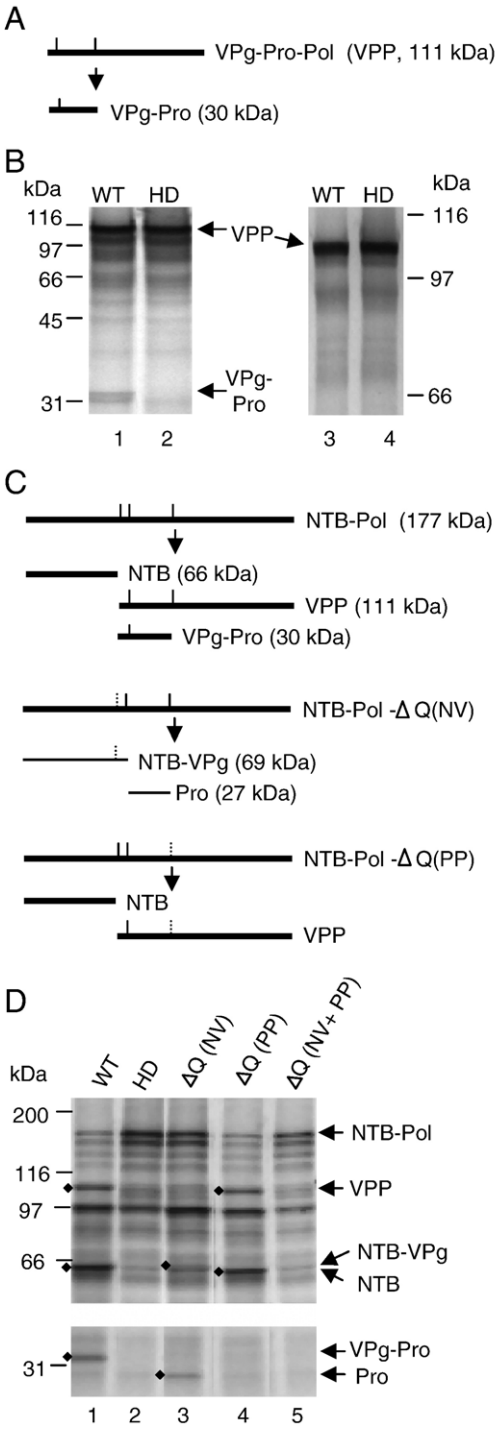


Fig. 2. *In vitro* processing of VPg-Pro-Pol and NTB-Pol: evidence that VPg-Pro-Pol is a stable precursor. (A) Schematic representation of the VPg-Pro-Pol precursor and of the VPg-Pro cleavage product produced after cleavage at the Pro-Pol site. Vertical lines represent cleavage sites within the polyprotein. (B) *In vitro* processing of the VPg-Pro-Pol precursor. Translation products of the wild-type (WT) VPg-Pro-Pol or of a derivative with a mutation in the proteinase catalytic triad (HD) were incubated at 16 °C for 16 h to allow proteolytic processing. Precursors and cleavage products were separated by SDS–PAGE (12% polyacrylamide for lanes 1 and 2 and 8% polyacrylamide for lanes 3 and 4) and visualized by autoradiography. Positions of the 111-kDa VPg-Pro-Pol polyprotein (VPP) and of the VPg-Pro cleaved product are indicated. Migration of the molecular mass standards is shown on the left of the gel for lanes 1 and 2 and on the right of the gel for lanes 3 and 4. (C) Schematic representation of the NTB-Pol precursor and of the cleavage products observed after proteolytic processing of the wild-type (WT) or mutated [ΔQ (NV) and ΔQ (PP)] precursors. Mutated cleavage sites are indicated by the vertical dashed lines. In the case of the NTB-Pol-ΔQ (NV) precursor, the cleavage was much less efficient, as shown by the thinner lines that represent the cleavage products. The NTB-Pol ΔQ (NV+PP) was not cleaved and is not shown in this panel. (D) *In vitro* processing of the NTB-Pol precursor. Translation products of the wild-type (WT) NTB-Pol or of derivatives with a mutation in the proteinase catalytic triad (HD), a deletion of the glutamine at the −1 position of the NTB-VPg cleavage site [ΔQ (NV)], a deletion of the glutamine at the −1 position of the Pro-Pol cleavage site [ΔQ (PP)] or a double deletion of the glutamines at the −1 position of the NTB-VPg and Pro-Pol cleavage sites [ΔQ (NV+PP)] were incubated at 16 °C for 16 h to allow proteolytic processing. Precursors and cleavage products were separated by SDS–PAGE and subjected to autoradiography. Positions of the NTB-Pol precursor and of various cleavage products are indicated by the diamonds at the left of each band and/or arrows on the right of the gel. Migration of the molecular mass standards is shown on the left of the gel.

Subcellular fractionation of the VPg-Pro-Pol′ polyprotein: a subpopulation of the protein is associated with ER-derived membranes

To study the distribution of the 95-kDa VPg-Pro-Pol′ polyprotein in different subcellular fractions prepared from ToRSV-infected cucumber cotyledons, post-nuclear fractions (S3) were centrifuged to obtain cytoplasmic (S30) and mem-

brane-enriched (P30) fractions. Probing these fractions in immunoblots revealed that the VPg-Pro-Pol′ polyprotein was present predominantly in the cytoplasmic fraction although a significant subpopulation of the protein was present in the membrane-enriched fraction (Fig. 3A).

We have previously shown that the ToRSV replication complex is associated with membranes derived from the ER (Han and Sanfacon, 2003). To determine if the subpopulation of VPg-Pro-Pol′ present in the membrane-enriched fraction (P30) is associated with ER-derived membranes, a concentrated P30 fraction was subjected to sucrose gradient fractionation as described previously (Han and Sanfacon, 2003). Experiments were conducted in the absence or presence of $MgCl_2$. It has been established that $MgCl_2$ preserves the integrity of the association of ribosomes with the rough ER. In the absence of $MgCl_2$, this association is disrupted and smooth ER is produced. This results in a shift of ER-containing fractions towards the top of the gradient (Wienecke et al., 1982). Two positive controls were used to identify fractions containing ER-derived membranes. The Bip protein is a soluble ER luminal protein. The ToRSV NTB and NTB-VPg proteins are integral membrane proteins

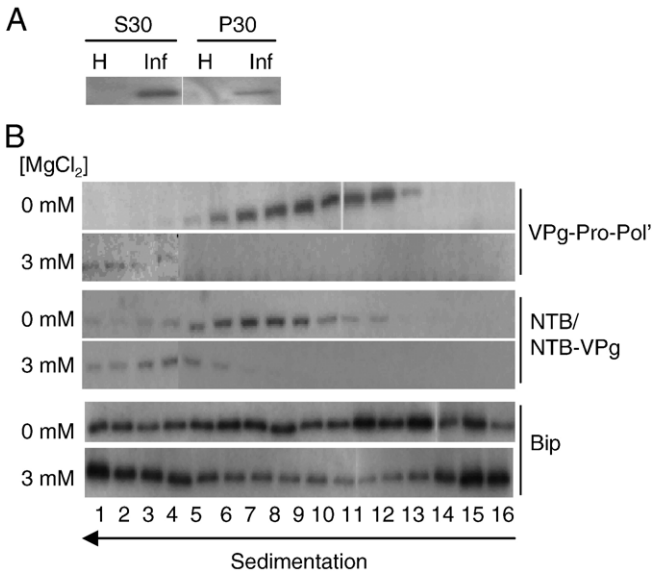


Fig. 3. Subcellular fractionation of the VPg-Pro-Pol′ polyprotein: a subpopulation of the protein is associated with ER-derived membranes. (A) The VPg-Pro-Pol′ polyprotein is distributed in both soluble and membrane-enriched fractions. ToRSV-infected (Inf) and healthy (H) cucumber tissue were fractionated into soluble (S30) and membrane-enriched (P30) fractions as described in Materials and methods. Proteins were separated by SDS–PAGE and the VPg-Pro-Pol′ polyprotein was detected by immunoblotting using anti-Pro antibodies. Only the relevant portion of the gel containing the VPg-Pro-Pol′ polyprotein is shown. (B) The VPg-Pro-Pol′ polyprotein present in the membrane-enriched fraction co-fractionates with ER-derived membranes. Proteins from the membrane-enriched fractions (P30 fraction shown in panel A) were fractionated on a 20–45% sucrose gradient in homogenization buffer in the absence and presence of $MgCl_2$.The direction of sedimentation is shown, with fraction 16 representing the top of the gradient. Proteins from each fraction were separated by SDS–PAGE, transferred to PVDF membranes and probed in immunoblots using antibodies against Pro, NTB and Bip. Only the relevant part of each immunoblot is shown. The blot probed with the NTB antibody shows the region containing the NTB and NTB-VPg proteins. The concentration of $MgCl_2$ used in each gradient is indicated on the left.

associated with ER-derived membranes active in replication (Han and Sanfacon, 2003). Fractions were collected and analyzed for the presence of VPg-Pro-Pol′, NTB, NTB-VPg and Bip. NTB and NTB-VPg co-fractionated with Bip (Fig. 3B). In the presence of 3 mM $MgCl_2$, the NTB, NTB-VPg and Bip proteins were present at the bottom of the gradient (fractions 1–6). Under these conditions, some of the Bip protein also partitioned to the top of the gradient. This phenomenon has been observed previously by us and by others (Han and Sanfacon, 2003; Schaad et al., 1997) and may be due to disruption of ER membranes during the extraction process, which would result in the release of the soluble luminal Bip. In the absence of $MgCl_2$, the NTB, NTB-VPg and Bip proteins shifted towards the top of the gradient (fractions 5–12), a result consistent with our previous observations and typical of ER-associated proteins (Han and Sanfacon, 2003). Interestingly, the behavior of the VPg-Pro-Pol′ polyprotein was very similar to that of the NTB and NTB-VPg proteins. In the presence of 3 mM $MgCl_2$, this protein was found at the bottom of the gradient. In the absence of $MgCl_2$, the VPg-Pro-Pol′ polyprotein shifted towards the top of the gradient and was found in fractions 6–12. We have previously shown that the NTB-VPg protein co-fractionates with the viral RdRp activity present in the bottom fractions of the sucrose gradients containing $MgCl_2$ (Han and Sanfacon, 2003). Taken together, these results suggest that a subpopulation of the VPg-Pro-Pol′ polyprotein is associated with ER-derived membranes that are active in viral replication.

The VPg, Pro and Pol domains do not associate with membranes in the absence of viral infection

Predictions based on computer-assisted analysis of the deduced amino acid sequence of the VPg, Pro and Pol domains suggested that these proteins do not contain membrane-targeting motifs (data not shown). To test if these proteins could associate with membranes when they were expressed individually, each domain was fused to the green fluorescent protein (GFP) and expressed in *Nicotiana benthamiana* by agroinfiltration. Extracts prepared from the agroinfiltrated leaves were separated into soluble (S30) and membrane-enriched (P30) fractions and probed with GFP antibodies in immunoblots. All fusion proteins were detected in the S30 fraction (Fig. 4A). Confocal microscopy analysis of the epidermal leaves revealed a similar subcellular distribution of the GFP fluorescence in cells expressing the free GFP or the various GFP fusion proteins (Fig. 4B). In all cases, the fluorescence was associated with the cytosol (which is compressed against the cell wall due to the presence of large vacuoles in plant cells) and diffused into the nucleus. The VPg-Pro and Pol domains were also fused to the HA tag to ensure that the GFP was not influencing the subcellular localization of the expressed fusion proteins. The HD mutation was introduced into the Pro domain of the VPg-Pro protein to eliminate *cis* cleavage between the VPg and Pro domains. Extracts were prepared as above and the presence of the proteins in the post-nuclear extract (S3), soluble (S30) and membrane-enriched (P30) fractions was tested by immunoblotting using anti-HA anti-

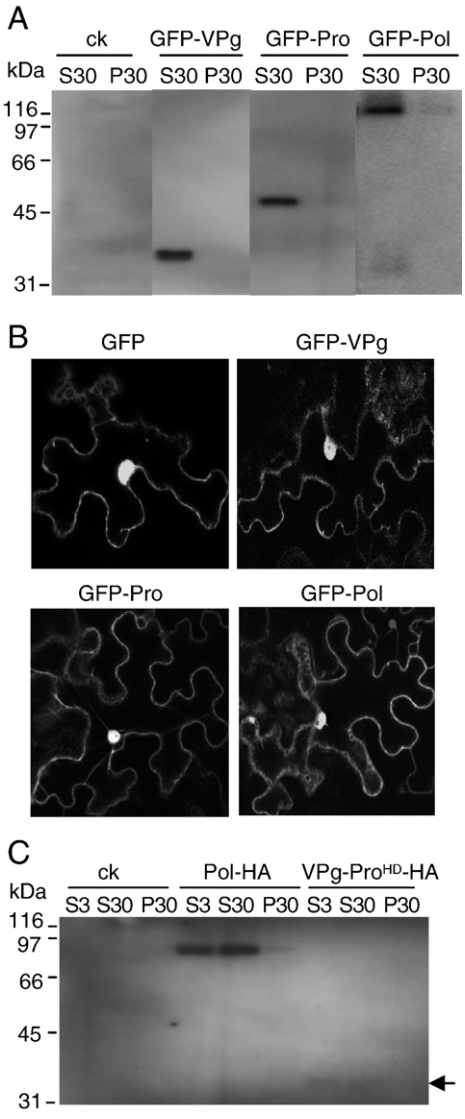


Fig. 4. The VPg, Pro and Pol domains do not associate with membranes in the absence of viral infection. (A) GFP fusion proteins containing the VPg, Pro or Pol domains fractionate in soluble fractions. The VPg, Pro and Pol domains were fused to GFP and expressed in *N. benthamiana* leaves by agroinfiltration in the presence of the p19 protein. Proteins were extracted and soluble (S30) and membrane-enriched fractions (P30) were produced as in Fig. 3. Proteins present in each fraction were separated by SDS–PAGE, transferred to PVDF membranes and probed with GFP antibodies. ck: control non-agroinfiltrated leaves. (B) Subcellular distribution of the GFP-VPg, GFP-Pro and GFP-Pol proteins is similar to that of the soluble free GFP protein. Leaf epidermal cells were examined by confocal microscopy 4 days after agroinfiltration. Entire cells are shown in each image. The GFP fluorescence (shown in white in the image) diffuses into the nucleus and is also present in the cytoplasm which is compressed against the cell wall due to the presence of a large vacuole in the cell. (C) Fusion proteins containing the VPg-Pro domains or the Pol domain fused to the HA epitope tag fractionate in soluble fractions. The VPg-Pro domains and the Pol domain were fused to the HA tag and expressed in *N. benthamiana* leaves by agroinfiltration. To ensure that proteolytic processing did not occur between the VPg and Pro domains in the VPg-Pro polyprotein, the proteinase was mutated in the catalytic triad (HD mutation). Cellular extracts were produced as above. S3: post-nuclear fraction; S30: fraction enriched in soluble proteins; and P30: fraction enriched in membrane proteins. Proteins were separated by SDS–PAGE and analyzed by immunoblotting as above but probed using anti-HA antibodies.

bodies. Similar to the fractionation of the GFP-fusions, the expressed HA-tagged proteins were also detected in the soluble fractions (Fig. 4C). These results indicate that the VPg, Pro and Pol proteins do not associate with membranes in the absence of viral infection and suggest that other viral proteins are necessary to promote the association of the VPg-Pro-Pol′ polyprotein with the membrane-bound replication complex in infected plant tissues.

The subpopulation of the VPg-Pro-Pol′ polyprotein detected in membrane-enriched fractions of ToRSV-infected cucumber extracts is peripherally associated with membranes

Since the VPg, Pro and Pol proteins do not target to membranes when expressed individually, experiments were conducted to look at the nature of the association of the VPg-Pro-Pol′ polyprotein with membranes in infected extracts. Peripherally associated membrane proteins can be distinguished from integral membrane proteins and luminal proteins by treatment of membrane-enriched fraction with various chemicals. Incubation with NaCl releases peripherally-associated proteins but not integral or luminal membrane proteins, while treatment with Na_2CO_3, pH 10.5 releases peripheral and luminal membrane proteins but not integral membrane proteins (Han and Sanfacon, 2003, and references therein). Membrane-enriched fractions were treated with either NaCl or Na_2CO_3 buffer and subsequently centrifuged at 30,000×*g* to distinguish between proteins that remain associated with membranes after the treatment (present in the new P30 fraction) and proteins that have been released from the membranes (present in the new S30 fraction). As above, Bip, a luminal ER protein, and NTB and NTB-VPg, two integral membrane proteins (Han and Sanfacon, 2003), were used as controls. Bip was present in higher concentration in extracts derived from infected plants than in those derived from healthy control plants (Fig. 5, compare lanes 9 and 10). This result was not surprising since ToRSV infection has previously been shown to induce proliferation of ER membranes (Han and Sanfacon, 2003). Treatment of the P30

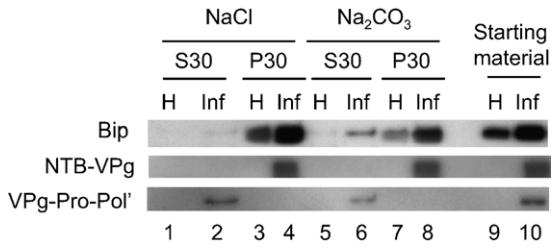


Fig. 5. The VPg-Pro-Pol′ polyprotein detected in membrane-enriched fractions of ToRSV-infected cucumber extracts is peripherally associated with membranes. The P30 fraction from healthy and ToRSV-infected plants (starting material, lanes 9 and 10) was extracted with either 1 M NaCl (lanes 1–4) or 0. 1 M Na_2CO_3, pH 10.5 (lanes 5–8) and then centrifuged at 30 000×*g* yielding S30 (lanes 1, 2, 5 and 6) and P30 (lanes 3, 4, 7 and 8) fractions. Proteins from equivalent amounts of these fractions were analyzed by SDS–PAGE followed by immunoblotting using anti-Bip antibodies (upper panel) to detect the Bip ER marker, anti-NTB antibodies (middle panel) to show the behavior of the NTB/NTB-VPg proteins and anti-Pro antibodies (lower panel) to detect the VPg-Pro-Pol′ polyprotein. Only the relevant portions of the gels are shown.

fraction with NaCl did not release the Bip protein or the NTB and NTB-VPg proteins from the membrane fraction (Fig. 5, lanes 1–4, top two panels). Treatment with Na_2CO_3 released a fraction of Bip into the S30 fraction as expected for a luminal membrane protein but did not release the NTB and NTB-VPg proteins (Fig. 5, lanes 5–8, top two panels). The VPg-Pro-Pol′ polyprotein was released from the membrane-enriched fraction by both the NaCl and Na_2CO_3 treatments (Fig. 5, bottom panel), confirming the suggestion that it is a peripheral membrane protein and that it is brought into the membrane-bound replication complex by protein–protein interactions.

Discussion

In this study, we report the detection of ToRSV replication proteins in infected cucumber plants using antibodies specific for the NTB, VPg, Pro and Pol domains. In addition to previously described proteins that contain the NTB and VPg domains (Han and Sanfacon, 2003), we have also detected a protein of 95 kDa that contains the VPg, Pro and Pol domains and probably corresponds to a truncated VPg-Pro-Pol intermediate polyprotein (Fig. 1). The simultaneous detection of several precursors that include the VPg domain indicates that ToRSV uses alternate processing pathways to produce its replication proteins *in vivo* (e.g., processing at the NTB-VPg cleavage site to release the VPg-Pro-Pol polyprotein and the mature NTB protein; and processing at the VPg-Pro site to release the NTB-VPg and X2-NTB-VPg polyproteins). In contrast, *in vitro* cleavage of various polyprotein precursors derived from the RNA1-encoded polyprotein (NTB-Pol, X1-Pro, X2-Pro and NTB-Pro) occurs predominantly at the NTB-VPg cleavage site (Fig. 2 and Wang and Sanfacon, 2000). In fact, cleavage at the VPg-Pro site is not detected *in vitro* unless the NTB-VPg cleavage site is mutated [Fig. 2, ΔQ (NV) mutant]. Although we cannot exclude the possibility that processing of partial polyprotein precursors differs from that of the entire RNA1-encoded polyprotein, a more likely explanation of our results is that *in vivo* processing of the RNA1-encoded polyprotein is influenced by cellular factors that are not present in the *in vitro* translation system. For example, we have previously shown that insertion of the NTB domain into ER membranes results in the translocation of the NTB-VPg cleavage site in the membrane lumen, which would probably prevent its recognition by the viral proteinase (Han and Sanfacon, 2003; Wang et al., 2004; Zhang et al., 2005). Similarly, alternative processing of the related *Cowpea mosaic virus* (CPMV, a comovirus) RNA1-encoded polyprotein has been suggested to explain the detection of both the 60K (NTB-VPg) and 112K (VPg-Pro-Pol) polyproteins in infected plants (Peters et al., 1992).

Alternative processing of polyproteins may be an efficient way for the virus to produce more than one form of the replication proteins and to regulate their activity at different stages of the replication cycle. The VPg protein plays a central role in replication. In picornaviruses, the VPg is uridylylated in the presence of the 3D protein (Pol) and has been shown to act as a primer for viral RNA replication (Paul et al., 1998). Similarly, the potyvirus VPg is uridylylated by the NIb-Pol (Puustinen and Makinen, 2004). It is likely that the VPg plays a similar role in nepovirus infection, although this needs to be demonstrated experimentally. In ToRSV-infected cells, the VPg domain is present in both the NTB-VPg and VPg-Pro-Pol polyproteins. In the NTB-VPg polyprotein, the VPg domain is translocated into the lumen of the membranes (Han and Sanfacon, 2003; Wang et al., 2004; Zhang et al., 2005) and is unlikely to be involved in priming RNA replication, a process which is presumably occurring on the cytoplasmic face of the membrane. In contrast, the VPg-Pro-Pol′ polyprotein detected in infected plants is peripherally associated with the cytoplasmic face of the membranes (Fig. 5). Thus, this polyprotein may serve as a donor for a VPg molecule active in RNA replication. In comoviruses, the equivalent 112-kDa polyprotein (VPg-Pro-Pol) has also been suggested to function as a VPg precursor *in vivo* (Peters et al., 1995). Processing of the ToRSV VPg-Pro-Pol polyprotein is very inefficient *in vitro* and probably occurs in two steps. First, it is cleaved at the Pro-Pol site to release the VPg-Pro intermediate (Fig. 2). Second, the VPg-Pro intermediate is processed into the mature VPg and Pro proteins (Chisholm et al., 2001). Both of these cleavages are suboptimal and occur slowly, suggesting that the release of the VPg from the VPg-Pro-Pol polyprotein is also an inefficient process *in vivo*. Similarly, in potyviruses, the replication-competent VPg is slowly released from the stable NIa polyprotein (VPg-Pro polyprotein) by cleavage at the suboptimal but essential VPg-Pro cleavage site (Carrington et al., 1993). Inefficient *in vitro* processing of the VPg-Pro-Pol intermediate has also been reported for two other nepoviruses (Hemmer et al., 1995; Margis et al., 1994).

The 95-kDa VPg-Pro-Pol′ polyprotein was associated with ER membranes active in viral replication (Fig. 3), suggesting that it may provide the RNA-dependent RNA polymerase activity. Accumulation of polyprotein precursors that contain the Pro and Pol domains in infected cells has been documented for a number of related viruses and many of these precursors have proteinase and/or polymerase activity. For example, a polyprotein that contains the Pro and Pol domains, and possibly also the VPg domain is detected in plants infected by another nepovirus (*Tomato black ring virus*, strain S, now classified as *Beet ringspot virus*) (Demangeat et al., 1992), although it is not known whether it is associated with the replication complex. In CPMV-infected cells, the 110K Pro-Pol polyprotein is the only viral protein present in purified RdRp preparations capable of elongating viral RNA and is probably the enzyme active in replication (Dorssers et al., 1984). The Pro-Pol precursor of *Feline calicivirus* (FCV) is the active RdRp (Wei et al., 2001) and in the MD145 norovirus, the Pro-Pol precursor has both proteolytic and polymerase activity (Belliot et al., 2005). The poliovirus (PV) 3CD (Pro-Pol) polyprotein accumulates in infected cells and play multiple roles in the viral replication cycle including sequestering the viral RNA from the translation complex to the replication complex, stimulation of viral RNA synthesis and proteolytic cleavage of the structural proteins (Franco et al., 2005, and references therein). However, it is not the active polymerase since this role is played by the mature 3D (Pol) (Franco et al., 2005).

Our results suggest that the 95-kDa VPg-Pro-Pol′ polyprotein detected in infected cucumber extracts is missing ~15 kDa from the C-terminus of the Pol domain. This is supported by the observation that the protein migrates faster on SDS–PAGE than the *in vitro* synthesized full-length VPg-Pro-Pol polyprotein. Since the N-terminal VPg domain of the VPg-Pro-Pol′ polyprotein is detected with the VPg antibodies, the missing fragment is likely from the C-terminus of the Pol domain. Although we cannot exclude completely the possibility that truncation of the full-length VPg-Pro-Pol polyprotein is caused by the release of plant proteases during the extraction process, this possibility seems unlikely since a set of protease inhibitors with a broad-range of specificity was added to the extraction buffer. We consider it more likely that

```
ToRSV  -SSVIKSLIQEAGVEERGVSKVGHIKDPAETPHVGGKTKLELVDEAFLVPSPVEVKIPSILSKDDPRIPEAYKGYDPLGDAMEKFYE 86
CPMV   GAEEYFDFLPAEENVSSGVAMVAGLKQGVYIPLPTKTALVETPSEWHLDTP--CDKVPSILVPTDPRIPAQHEGYDPAKSGVSKYSQ 85
PV     -----GEIQWMRPSKEVGYPIIN----------APSKTKLEPSAFHYVFEG---VKEPAVLTKNDPRLKTDFE-----EAIFSKYVG 64
FCV    ------VTAQKYDVTKPDISYKGLICKQLDEIRIIPKGTRLHVSPAHTEDFEECSHQPASLGSGDPRCPKSLT------AIVVDSLK 75
                     . . .                .         .        : *: *   ***              . .

ToRSV  PMLDLDEDVLESVMADMYDEFYDCQTTLR---IMSDDEVINGSDFGFNIEAVVKGTSEGYPFVLSRRPGEKGKARFLEELEPQPGD 169
CPMV   PMSALDPELLGEVANDVLELWHDCAVDWDDFGEVSLEEALNGCEGVEYMERIPLATSEGFPHILSRNGKEKGKRRFVQGDD----- 166
PV     NKITEVDEYMKEAVDHYAGQLMSLDINTE---QMCLEDAMYGTDG---LEALDLSTSAGYPYVAMGKKKRD--------------- 129
FCV    PYCDKVEGPPHDILHRVQKMLIDHLSGFVPVNISSETSMLS------AFHKLNHDTSCG-PYLGGRKKDHMTNG------------ 142
                .          .           .  . :        :. :   ** * *.:   .  .

                                                  I                          II
ToRSV  TKPKYKLVVGTEVHSAMVAMEQQARTE--VPLLIGMDVPKDERLKPSKVLEKEKTRTFVVLPMHYNLLLRKYVGILCSSMQVNRH- 250
CPMV   --CVVSLIPGTTVAKAYEELEASAHRF--VPALVGIECPKDEKLPMRKVFDKEKTRCFTILPMEYNLVVRRKFLNFVRFIMANRH- 246
PV     -----ILNKQTRDTKEMQKLLDTYGIN--LPLVT---YVKDELRSKTKVEQG-KSRLIEASSLNDSVAMRMAFGNLYAAFHKNFGV 203
FCV    -------EPDKPLLDLLSAKWKLATQGIALPHEYTI-GLKDELRPVEKVAEG-KRRMIWGCDVGVATVCAAAFKGVSDAITANHQ- 217
             :   .   .              :*        ***     **    * * :    :          .  .   :  *

             III                              IV
ToRSV  RLACAVGTNPYSRDWTDIYQRLAEKNSVALNCDYSRFDGLLNYQAYVHIVNFINKLYN--DEHSIVRGNLLMAMYGRWSVCGQRVF 337
CPMV   RLSCQVGINPYSMEWSRLAARMKEKGNDVLCCDYSSFDGLLSKQVMDVIASMINELCGGEDQLKNARRNLLMACCSRLAICKNTVW 334
PV     ITGSAVGCDPD-LFWSKIPVLMEEK---LFAFDYTGYDASLSPAWFEALKMVLEKIGFG------DRVDYIDYLNHSHHLYKNKTY 281
FCV    YGPVQVGINMDSPSVEALHQRIKSAAK-VYAVDYSKWDSTQSPRVSAASIDILRYFSDR---SPIVDSAANTLKSPPIAIFNGVAV 301
        .  .**    .     :   : .        **: :*.  .         .:. :                        :    .

                V                                          VI
ToRSV  EVRAGMPSGCALTVIINSLFNEMLIRYVYR-----ITVPRPLVNNFKQEVCLIVYGDDNLISIKPDTMKYFNGEQIKTILAKYKVT 417
CPMV   RVECGIPSGFPMTVIVNSIFNEILIRYHYKKLMREQQAPELMVQSFDKLIGLVTYGDDNLISVNAVVTPYFDGKKLKQSLAQGGVT 419
PV     CVKGGMPSGCSGTSIFNSMINNLIIR---------TLLLKTYKGIDLDHLKMIAYGDDVIASYP----HEVDASLLAQSGKDYGLT 353
FCV    KVSSGLPSGMPLTSVINSLNHCLYVGCAILQS--LEARGVPVTWNLFSTFDMMTYGDDGVYMFP--MMFASVSDQIFANLSAYGLK 382
        *  *:*** . * :.**: : : :                          . . ::.**** :            .. :        :.

                          VII
ToRSV  ITDGSDKNSPVLRAKPLKQLDFLKRGFRVESDGRVLAPLDLQAIYSSLYYINPQGNILKSLFLN-AQVALR-----ELYLHG--DV 495
CPMV   ITDGKDKTSLELPFRRLEECDFLKRTFVQRSSTIWDAPEDKASLWSQLHYVNCNNCEKEVAYLTNVVNVLR-----ELYMHSPREA 500
PV     MTP--ADKSATFETVTWENVTFLKRFFRADEK-------YPFLIHPVMPMKEIHESIRWTKDPRNTQDHVR-----SLCLLAWHNG 425
FCV    PTR-VDKSVGSIEPIDPESVVFLKRTITRTPQG-IRGLLDRSSIIRQFYYIKGENSDDWKTPPKSIDPTSRGQQLWNACLYASQHG 466
        *    ..   :     :.  **** :    .           :   :   : .               *     .  : .  .

ToRSV  EQFTAVRNFYVNQIG-GNFLSLPQWRHCASFHDEQYSQWKPWSPVKFLEVDVPDAKFLQHKAPAT----ALSIVADRLAVAGPGWR 576
CPMV   TEFRRKVLKKVSWITSGDLPTLAQLQEFYEYQRQQGGADNNDTCDLLTSVDLLGPPLSFEKEAMHGCKVSEEIVTKNLAYYDFKRK 586
PV     EEEYNKFLAKIRSVPIGRALLLPEYSTLYRRWLDSF---------------------------------------------------- 461
FCV    VEFYNKIYKLAQKAVEYEELHLEPP--TYHSALEHYNNQFNGVEARSDQIDSSGMTALHCDVFEV---------------------- 529
        :                    *           :

ToRSV  NKDPDRYLLVSLTSLKAN-EGGLYFPVDYGEGTGQQATEASIRAYRRLKDHRVRHMRDSWNEGKTIVFRCEGPFVSGWAAAISFGT 661
CPMV   GEDEVVFLFNTLYPQSSLPDGCHSVTWSQGSGRGGLPTQSWMSYNISRKDSNINKIIRTAVSSKKRVIFCARDNMVPVNIVALLCA 672
PV     ---------------------------------------------------------------------------------------
FCV    ---------------------------------------------------------------------------------------

ToRSV  SVGMNAQDLLINYGIQGGAHKEYLGRYFVGARFKELERYDRPFQSRIIAS 711
CPMV   VRNKLMPTAVSNATLVKVMENAKAFKFLP-------EEFNFAFSFSDV-- 711
PV     --------------------------------------------------
FCV    --------------------------------------------------
```

Fig. 6. Alignments of Pol domains from *Tomato ringspot virus*, *Cowpea mosaic virus*, poliovirus and feline calicivirus. The deduced amino acid sequence of the Pol domains from ToRSV, *Cowpea mosaic virus* (CPMV), poliovirus (PV) and feline calicivirus (FCV) were aligned using the Clustal W program. Previously recognized conserved motifs (reviewed by Koonin and Dolja, 1993) are boxed and labeled I to VII.

truncation of the C-terminal region of Pol is a specific cleavage event occurring during the course of ToRSV infection. Interestingly, the Pol domain of nepoviruses and comoviruses (80–82 kDa) is larger than the Pol domain of picornaviruses, caliciviruses and potyviruses (50–58 kDa). Alignments of the Pol domains of ToRSV, CPMV, PV and FCV using the Clustal W program (Thompson et al., 1994) revealed that while the N-terminal 60 kDa of the ToRSV and CPMV Pol align with the Pol of PV and FCV and includes all the conserved RNA-dependent RNA polymerase motifs (Fig. 6), the C-terminal 20 kDa is apparently unique to the family Comoviridae. Further experiments aimed at comparing the polymerase activity of the truncated 95-kDa VPg-Pro-Pol′ polyprotein, the full-length 111-kDa VPg-Pro-Pol polyprotein and the mature 81-kDa Pol should provide valuable information on the biological relevance of the various forms of the polymerase. The nature of the cleavage leading to the release of a truncated form of the ToRSV VPg-Pro-Pol in infected cucumber plants is not known. Cleavage within the Pol domain by the ToRSV proteinase was not observed *in vitro* (Fig. 2). Additional work will be necessary to determine whether the C-terminal domain of the VPg-Pro-Pol polyprotein is cleaved by a plant proteinase or whether cellular factors enhance the recognition of this cleavage site by the viral proteinase.

The VPg-Pro-Pol′ polyprotein was detected in both the cytosolic and membrane fractions. There are two possible interpretations for this result. First, the portion of VPg-Pro-Pol′ detected in the S30 fraction may have been accidentally released from the membranes during the extraction process. Second, two subpopulations of VPg-Pro-Pol′ may co-exist in ToRSV-infected cells. While it is likely that the membrane-associated VPg-Pro-Pol′ is involved in viral RNA replication, the biological function of a cytoplasmic subpopulation of the VPg-Pro-Pol′ is not known. One attractive possibility is that it possesses proteolytic activity directed at one or several cellular factors as has been shown for the 3CD polyprotein of poliovirus (Roehl et al., 1997). We have suggested above that the peripheral association of the ToRSV VPg-Pro-Pol′ polyprotein with ER-derived membranes is mediated by its interaction with one or several viral membrane proteins. Studies are underway to determine whether the two potential membrane anchors for the replication complex, the NTB-VPg intermediate and the X2 protein (Han and Sanfacon, 2003; Zhang and Sanfacon, 2006; Zhang et al., 2005), play a role in this process.

Materials and methods

Construction of plasmids

Plasmid pBIN-GFP-VPg was constructed by first amplifying the VPg region from plasmid pMR10 (Rott et al., 1995) with Pfu polymerase (Stratagene) and primers G5 and G6 (see Table 1 for a list of primers). The amplified product was digested by *Sst*I and *Kpn*I and ligated with the large *Sst*I–*Kpn*I fragment of plasmid pBIN-GFP-nN (Zhang et al., 2005).

Table 1
Primers used in plasmid constructions

No.	Sequence (5′ to 3′)[a]	Comments[b]
G5	ttatagagctc**ggtggcggatca**tcgacgattccctccggtag	nts 3713–3733 + *Sst*I + GGGS (+)
G6	gcgcgcggtacc**tta**ctgtacagattgtgggcgg	nts 3794–3780 + *Kpn*I + taa (−)
G7	atatgagctc**ggtggcggatca**ggttcttctttggcggaagc	nts 3795–3816 + *Sst*I + GGGS (+)
G8	gcgcgcggtacc**tta**ctggcaaggagcaaaagaag	nts 4535–4516 + *Kpn*I + taa (−)
G9	ttatatgagctc**ggtggcggatca**tctagtgttatcaaat ctctaattcaagagg	nts 4536–4567 + *Sst*I + GGGS (+)
G10	ccgcgcggtacc**tta**gctcgcaattatacgagactg	nts 6668–6648 + *Kpn*I + taa (−)
28	acgcccatgggaattaagtgctgagttgt	nts 2948–2967 + NcoI (+)
35	acgcgtcgacggcagctactcgcct	nts 6696–6682 + *Sal*I (−)
85	cgggatcctatctagtgttatcaaatctcta	nts 4537–4556 + *Bam*HI (+)
86	cgggatcctatctagtgttatcaaatctcta	nts 6611–6591 + *Xho*I (−)
96	acgac**atg**gccacgacgattccctccggtag	nts 3715–3733 + *Msc*I + atg (+)
97	cagcatctcgag**tta**gctcgcaattatacgagactg	nts 6671–6648 + *Xho*I + taa (−)
112	gctctag**atg**tcgacgattccctccggtag	nts 3713–3734 + *Xba*I + atg (+)
114	ggggtacc**ttacgcatagtcaggaacatcgtatgggta** ctggcaaggagcaaaagaag	nts 4535–4517 + Kpn I + taa + HA (−)
115	gctctag**atg**tctagtgttatcaaatctctaattcaagagg	nts 4536–4566 + *Xba*I + atg (+)
117	ggggtacc**ttacgcatagtcaggaacatcgtatgggta** gctcgcaattatacgagactg	nts 6668–6648 + *Kpn*I + taa + HA (−)

[a] Introduced restriction sites are underlined. Insertion of amino acid spacer, HA epitope or stop (taa) or start (atg) codon is shown in bold.

[b] For each plasmid, the region homologous to ToRSV sequence is indicated (e.g. specific nts of the ToRSV RNA1 sequence). Additional features are also indicated including: introduced restriction sites, introduced start or stop codons (atg or taa), introduced HA epitope (HA) or linker amino acid sequences (GGGS, in the one-letter code). Finally, the strand to which the primer corresponds (+ for coding strand and − for non-coding strand) is also indicated.

This strategy was also used to obtain plasmids pBIN-GFP-Pro using primers G7 and G8 and pBIN-GFP-Pol using primers G9 and G10. Plasmid pBIN-VPg-Pro^{HD}-HA was constructed by amplifying the VPg-Pro^{HD} region from plasmid pT7-VPg-Pro^{H1283D}-N-Pol-II (Wang et al., 1999) using primers 112 and 114 and Pfu polymerase. The amplified product was digested with *Xba*I and *Kpn*I and cloned into the corresponding sites of intermediate plasmid pBIN-cNV$_3$-HA (Zhang et al., 2005) for agroinfiltration. A similar strategy was used to make clone pBIN-Pol-HA using the same template and primers 115 and 117. To construct plasmid pET21d-Pol, a PCR product containing almost the entire coding region for the Pol domain was amplified using plasmid pMR10 as the template and primers 85 and 86. The amplified product was digested with *Bam*HI and *Xho*I and inserted into the corresponding sites of plasmid pET21d (Novagen), resulting in an in frame fusion with the His tag. Plasmid pCITE-VPg-Pro-Pol was constructed by amplifying the entire coding region for the VPg, Pro and Pol domains using

primers 96 and 97 and Pfu polymerase. The resulting product was digested with *Msc*I and *Xho*I and ligated into the corresponding sites of plasmid pCITE 4a (Novagen). Plasmid pCITE-VPg-Pro^{H1283D}-Pol was generated by digesting plasmid pT7-PRO^{H1283D} (Hans and Sanfacon, 1995) with *Pst*I and *Bam*HI and exchanging the excised fragment into the corresponding sites of plasmid pCITE-VPg-Pro-Pol. To construct plasmid pCITE-NTB-Pol, the coding region for the C-terminal half of NTB and for the entire VPg, Pro and Pol domains was amplified using primers 28 and 35 and Pfu polymerase. The resulting product was digested by *Bam*HI (an internal site located in the C-terminal region of the Pro domain) and *Sal*I and inserted into the corresponding sites of previously described plasmids pCITE-NTB-Pro or pCITE-NTB-Pro^{H1283D} (Wang et al., 1999) to produce plasmid pCITE-NTB-Pol and pCITE-NTB-Pol^{H1283D}. Precise deletions of the conserved glutamine at the −1 position of the NTB-VPg (Q^{1212}) and/or Pro-Pol (Q^{1486}) cleavage sites were generated by site-directed mutagenesis using specific primers as previously described (Carrier et al., 1999).

Recombinant polymerase expression and antibody production

For expression of pET21d-Pol, the plasmid was transformed into BL21 (DE3) cells (Novagen) following the supplier's instructions. Cells were grown at 37 °C to an OD_{600} of 0.5. Then expression was induced by adding 1 mM IPTG and continuing the culture for 2 h at 37 °C. The recombinant polymerase was purified from the bacteria using Ni-NTA resin (Qiagen). Purity of the recombinant protein was assessed by SDS–PAGE analysis. Purified preparations of the polymerase were used for rabbit polyclonal antibody production. Similarly, polyclonal antibody was prepared against the recombinant proteinase that was prepared as previously described (Chisholm et al., 2001). The specificity of the antibodies was confirmed by immunoblotting using purified recombinant proteinase and polymerase or plant extracts expressing GFP-Pol and GFP-Pro fusions (data not shown).

Preparation of plant extracts and subcellular fractionation

Cucumis sativus var. Straight Eight plants were inoculated with ToRSV inoculum 1 week after planting. Inoculum was prepared by extracting ToRSV-infected cucumber plants 1:4 (w/v) in 50 mM sodium phosphate buffer, pH 7.2. Cotyledons were sprayed with carborundum before the inoculum was manually applied. Four to 6 days after inoculation, cotyledons showing symptoms were extracted to obtain the S3 or S30 and P30 fractions essentially as described previously (Han and Sanfacon, 2003) except that Complete protease inhibitor (Roche) was substituted for leupeptin and aprotinin in the homogenization buffer. Briefly, the post-nuclear fraction (S3 fraction) was obtained by centrifugation of the plant extracts at 3,700×*g* for 10 min at 4 °C. Centrifugation of the S3 fraction at 30,000×*g* for 20 min at 4 °C was used to prepare the membrane-enriched and cytoplasmic fractions (pellet and supernatant fractions or P30 and S30 fractions, respectively). To allow meaningful comparisons of the protein concentration in each fraction, P30 fractions were resuspended in a volume of buffer equal to that of the S30 fraction. A concentrated P30 fraction from healthy and ToRSV-infected tissue was prepared and loaded onto sucrose gradients (20–45% sucrose in homogenization buffer) as described (Schaad et al., 1997). The extraction and sucrose gradient fractionation were conducted in the presence or absence of 3 mM $MgCl_2$. Samples were centrifuged and fractions were collected and analyzed by immunoblotting (Han and Sanfacon, 2003).

Agroinfiltration of N. benthamiana plants and confocal microscopy

Binary vectors containing the plant expression cassettes with the GFP or HA fusions were transformed into *Agrobacterium tumefaciens* LBA4404 (Invitrogen) by electroporation. Cultures were used for agroinfiltration in the presence of the *Tomato bushy stunt virus* p19 suppressor of gene silencing as previously described (Zhang et al., 2005). Three to 4 days after agroinfiltration, leaves were extracted (Zhang et al., 2005) and GFP fluorescence was analyzed by confocal microscopy (Leica). The acquired images were processed using Leica software and Photoshop 7.0 (Adobe).

SDS–PAGE and immunoblotting

Proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and detected in immunoblots using anti-Pro, anti-Pol, anti-Bip (donated by M. Chrispeels), anti-NTB (Han and Sanfacon, 2003) and anti-VPg (Wang et al., 1999) polyclonal antibodies and anti-GFP (BD Biosciences) and anti-HA (clone 3F10, Roche) monoclonal antibodies. The secondary antibodies were goat anti-rabbit-HRP for the polyclonal antibodies, goat anti-mouse-HRP for the GFP monoclonal antibody and goat anti-rat-HRP for the HA monoclonal antibody (Medicorp). Membranes were developed using an ECL development kit (GE Healthcare).

Membrane protein extraction

Crude membrane-enriched fractions (P30) were resuspended in either 1 M NaCl or 0.1 M Na_2CO_3, pH 10.5 and incubated on ice with occasional mixing for 30 min (Han and Sanfacon, 2003). The samples were then centrifuged at 30,000×*g* for 30 min at 4 °C. The pellet fraction was resuspended in a volume of buffer equal to that of the supernatant and the proteins were analyzed by SDS–PAGE and immunoblotting as described above.

In vitro translations

Coupled *in vitro* transcription and translation reactions and *in vitro* processing assays using wild-type or mutated plasmids pCITE-VPg-Pro-Pol and pCITE-NTB-Pol were conducted as described previously (Wang et al., 1999). Labeled proteins were visualized by autoradiography.

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